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Technical Note: Comparison of Chromatographic Profile of Glycomacropeptide from Cheese Whey Isolated Using Different Methods

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ABSTRACT

Glycomacropeptide (GMP) has heterogeneous carbohydrates, and this attributes to its various biological activities. In this study, we compared the chromatographic profiles of GMP isolated by three methods (trichloracetic acid fractionation, ethanol precipitation, and ultrafiltration) from whey protein isolate (WPI). Seven sharp heterogeneous GMP peaks were eluted from GMP prepared by ethanol precipitation and ultrafiltration using Mono Q anionic chromatography, while only 5 peaks were seen in TCA treated sample. The TCA pretreatment recovered only sialo-GMP (glycosylated) and eliminated all contaminated proteins; however, the recovery rate was the lowest (6.7% of the initial WPI). Ethanol precipitation recovered 20.4% of GMP from WPI and 75.7% was glycosylated, but the heating process might lead to degradation of glycosidic residues. Ultrafiltration was found to be the most effective in recovering GMP. The recovery rate was 33.9% with 81.6% sialo-GMP. We concluded that carbohydrate profile of GMP varied widely and depended on the isolation method. Based on the high recovery of sialo-GMP, the combination of ultrafiltration and anionic chromatography might be a suitable and practical approach on an industrial scale.
(**Key words:** glycomacropeptide, whey protein isolate, carbohydrate, HPLC)

Abbreviation key: GMP = glycomacropeptide, **UF** = ultrafiltration, **WPI** = whey protein isolate.

Glycomacropeptide (**GMP**) present in cheese whey is a C-terminal glycopeptide released from κ-CN by the action of chymosin at ^{105}Phe-^{106}Met during cheese making (Eigel et al., 1984). It is the sole milk glycopeptide and contains various amounts of covalently attached oligosaccharides, including *N*-acetylneuraminic acids (sialic acids), galactose, and *N*-acetylgalactosamine (Saito et al, 1991). Many researchers have discovered that GMP exhibits various biological activities, such as suppression of gastric secretion, promotion of bifido-bacterial growth, binding of cholera and *Escherichia coli* enterotoxin, inhibition of viral and bacterial adhesion, and modulation of immune system (Brody, 2000). The multifunctional bioactivities of GMP might be attributed to the carbohydrate chains, but the degree to which the carbohydrate moiety remains attached to GMP and its role in the biological functions of GMP has not been completely revealed (Dziuba and Minkiewicz, 1996). Because of its unique carbohydrate composition and biological activities, GMP is thought to be a potential ingredient for functional foods and nutraceuticals, and thus much attention has been given to the development of techniques to isolate and purify GMP from cheese whey. Glycomacropeptide has been isolated and purified from cheese whey or rennet casein by using various methods, such as TCA pretreatment (Morr and Seo, 1988), ethanol precipitation (Saito et al., 1991), and ultrafiltration (Kawasaki et al., 1993; Chu et al., 1996). Although many of GMP isolation methods have been described, few commercially viable technologies have been reported (Xu et al., 2000), and the effect of different isolation methods on GMP carbohydrate profiles has not yet been evaluated. Our study was thus undertaken to compare the above-three isolation methods of GMP from whey protein isolate (**WPI**) on the effect of its carbohydrate profile and to provide an effective isolating method that is suitable for industrial needs.

MATERIALS AND METHODS

Isolation of GMP using a TCA pretreatment was followed by the procedures described by Morr and Seo (1988). Twenty milliliters of 5% (wt/vol) WPI (Protein Fractionation Inc, Toronto, Canada) was dissolved in 12% (vol/vol) TCA. Precipitate was removed by centrifuge at 5000 × *g* for 20 min. Supernatant that contained GMP was then dialyzed against deionized water for 2 d with 3.5-kDa cut-off membrane (Fisher Scientific,

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Nepean, Ontario, Canada). In ethanol precipitation method, 20 mL of 5% WPI (pH 3.5) was heat at 98°C for 1 h, followed by rapid cooling to 4°C, and precipitated with 50% (vol/vol) cold ethanol. The solution was then readjusted to pH 9.0 by 1.0 *N* NaOH, and flocculent material was removed by centrifuge at 3000 × *g* for 30 min, leaving a supernatant containing GMP (Saito et al., 1991). Ultrafiltration was also documented to isolate GMP with a modification of the method described before (Kawasaki et al, 1993). Twenty milliliters of 5% WPI at pH 3.5 was filtered through Whatman No. 1 filter (Fisher Scientific), and then subjected to Amicon 8050 ultrafiltrater (Millipore Corporation, Bedford, MA) fitted with 50-kDa MW cut-off ultrafiltration membrane (Millipore Corporation). After the first ultrafiltration, permeate was readjusted to pH 7.0 with 1.0 *N* NaOH. The second and third ultrafiltration was carried out by the same ultrafiltration (**UF**) membrane to retain GMP in the retentate.

All crude GMP were then subjected to Mono Q HR5/5 anion-exchange chromatography (Pharmacia Biotech., Uppsala, Sweden) with a linear gradient of NaCl from 0 to 1.0 *M*, using HPLC (Waters, Ventura, CA) to analyze the heterogeneity of each GMP component. The column was equilibrated and eluted with 20 m*M* Tris-HCl buffer at pH 8.0 at flow rate of 1.0 mL/min. The effluent was monitored at 214 nm.

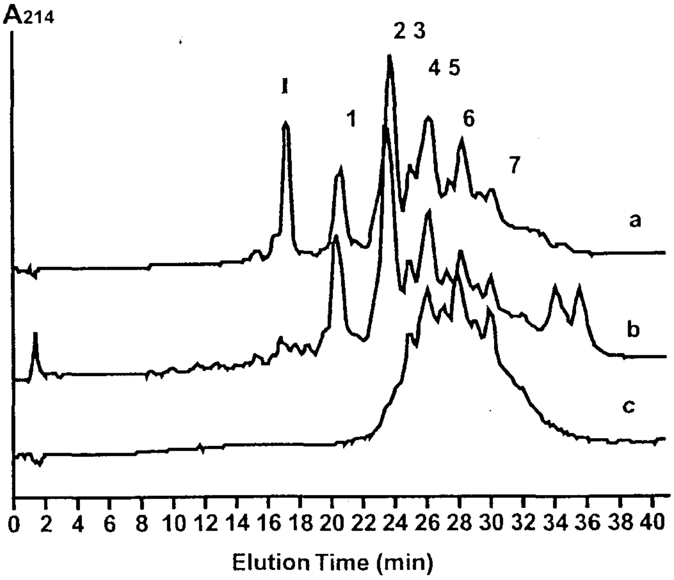

Figure 1. Elution profile of glycomacropeptide isolated from whey protein isolate by (a) ultrafiltration, (b) ethanol precipitation, and (c) TCA pretreatment under Mono Q HR5/5 anionic HPLC in 20 m*M* Tris-HCl buffer at pH 8.0. I; impurity.

RESULTS AND DISCUSSION

Glycomacropeptide could be recovered from WPI by all three methods described above. The apparent molecular weight was found to be 20 to 24 kDa from 15% SDS polyacrylamide electrophoresis (data not shown). Although it was far above the theoretical MW of 7 kDa, the apparent molecular weight of GMP was comparable to those reported previously. According to a previous study of GMP by gel chromatography, molecular weight of GMP aggregate was pH dependent. Molecular weight was distributed from 20 to 50 kDa at pH 7 and from 10 to 30 kDa at pH 3.5 (Kawasaki et al., 1993). The heterogeneous sugar chains that attached to the GMP molecule also contributed to the apparent molecular weight of GMP. Several studies have reported that at least 5 heterogeneous sugar chains were identified from bovine GMP (Flat et al., 1972; Saito et al, 1992). The biological activities of GMP are thought to depend on the content and structure of these sugar moieties (Dziuba et al., 1991).

Figure 1 illustrated the HPLC profile of GMP prepared by TCA pretreatment, ethanol precipitation, and UF. Seven sharp GMP peaks were eluted from GMP prepared by ethanol precipitation and UF, whereas only 6 peaks were identified in the TCA-treated sample. The multiple elution peaks could be due to the heterogeneous sugar chains. This result was consistent with previous reports (Saito et al., 1991). The first peak was previously identified as an asialo-GMP (nonglycosylated), and followed by 6 heterogeneous sialo-GMP (glycosylated) peaks (Kawakami et al., 1992). Table 1 summarized the percentage of each peak to the total recovered GMP isolated by different methods with different carbohydrate compositions in each GMP peak fraction. Because these heterogeneous sugar chains were thought to be responsible for various biological activities of GMP, high recovery of sialo-GMP would be of great interest for nutraceutical research.

The TCA pretreatment successfully isolated carbohydrate-rich GMP fractions. All of the recovered GMP was glycosylated. Although no other contaminant peaks were observed in TCA pretreated GMP, only 5 blurred peaks were eluted. However, the recovery rate was the lowest among the three, only 6.7% of the initial WPI. The recovery rate of GMP was calculated relative to the initial protein concentration (GMP concentration/initial WPI concentration) (Kawasaki et al., 1993). The TCA treatment eliminated asialo-GMP and the first sialo-GMP fraction, probably because TCA could precipitate small sugar chains due to their low hydropilicity. It was reported that the different asialo- and sialo-GMP have different sensitivities to TCA precipitation (Leonil and Molle, 1991). In addition, the presence of the large TCA peaks precluded the visual-

Table 1. Percentage of glycomacropeptide (GMP) fractions by different isolation methods from whey protein isolate.

Peak	1	2	3	4	5	6	7	Total glycosylated GMP
Method: (% of each peak/total GMP)[1]								
UF	18.4	46.3	3.2	17.6	1.1	9.5	4.0	81.7
EtOH	24.3	46.2	3.1	14.1	1.3	7.7	3.3	75.7
TCA	—	—	12.1	23.1	5.0	36.2	23.5	100
Carbohydrate and phosphorus content (mol/mol)[2]								
NeuAc	0	0	1	2	3	4	5	
Gal	0	1	1	2	2	2	3	
GalNAc	0	1	1	2	2	2	3	
Phosphorus	0	1	1	1	1	1	1	

[1]The data are means of three repetitions. UF; ultrafiltration, EtOH; ethanol precipitation, TCA; trichloroacetic acid fractionation. NeuAc = *N*-acetylneuraminic acid (sialic acid); Gal = galactose and GalNAc = *N*-acetylgalactosamine.

[2]Data were adapted by Kawakami et al., 1992.

ization of possible peptides that might be eluted in the same retention time. Thus, the pretreatment of whey with TCA might lead to poor estimation of GMP content unless the differential solubility of GMP in TCA was taken into consideration (Kawakami et al., 1992).

Ethanol precipitation recovered both asialo- and sialo-GMP simultaneously. Glycomacropeptide (20.4%) was recovered, and 75.7% was glycosylated. Although this method had a reasonably high recovery and purity of sialo-GMP, heat coagulation of whey protein during the isolation process might lead to partial hydrolysis of κ-CN with liberation of GMP and degrade glycosidic residues (van Hooydonk et al., 1987). Heat coagulation degraded some of the sugar chains in sialo-GMP and gave rise to a higher ratio of asialo-GMP (Table 1).

Among the 3 isolation methods, UF was found to have the highest recovery of GMP (33.9%) with 81.6% sialo-GMP. Ultrafiltration provided the most convenient and effective way to eliminate the pretreatment of whey with TCA and ethanol, while providing genuine content of both asialo- and sialo-GMP simultaneously. In fact, membrane filtration is the most widely used method presently on industrial basis for recovery of whey proteins such as α-lactalbumin and β-LG (Rossano et al., 2001).

In conclusion, different isolation and preparation methods of GMP resulted in different chromatographic profile in GMP. These heterogeneous carbohydrate chains were the unique characteristics of GMP, not found in the remaining part (1 to 105) of κ-CN. Each of the GMP fractions with different carbohydrate chains might be responsible for different biological functions. The result obtained from this study provides useful information for further investigation as to structure-function studies of biological activities of GMP. In addition, UF method was found to be the most effective in GMP separation compared with TCA and ethanol pretreatment method. Not only of its high recovery and high purity of sialo-GMP, it also eliminated the pretreatment of sample with TCA and ethanol while retaining the structure of the carbohydrate structures. The combination of UF and an anionic chromatography may be suitable and practical for industrial production of GMP.

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